

THE CONTRACTILE VACUOLE AND THE ADJUSTMENT TO CHANGING CONCENTRATION IN FRESH WATER AMOEBAE

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INTRODUCTION

In considering the relationship existing between an organism and its environment, it is necessary to distinguish clearly between the environment of the organism and the environment of the individual cells. The effect of the environment on an organism will depend specifically on the extent to which the cellular environment is controlled by the organism as a whole. As pointed out by Krogh (1939), the osmotic environment of the tissue cells of the vertebrates—the body fluid—is maintained at a very constant level. This is true to a lesser degree among the lower organisms. In the free-living protozoa, the cellular environment is the outside environment of the organism and is controlled little, if at all, by the organism. We find consequently that the individual cells of higher organisms have lost their resistance to osmotic changes while the cells of many of the protozoa, of necessity, have retained the ability to withstand considerable variation in the osmotic environment. It is well known that certain bacteria and molds can withstand enormous osmotic changes—which also have been shown to be true of a considerable number of protozoa (Hopkins, 1938; Butts, 1935; Mast and Hopkins, 1941; and Kitching, 1938).

The nature of the adjustments made by cells capable of withstanding great osmotic changes is not clearly understood. In the literature, the assumption is rather generally made that the osmotic concentration of the cellular contents must be maintained at a fairly constant value in order to be consistent with the metabolic processes. This assumption is made, regardless of the fact that evidence for the constancy of the intracellular osmotic pressure is conspicuously absent, especially for the lower organisms. We find that the prevailing opinion as to the function of the contractile vacuole of the protozoa is that it is an organelle which operates to maintain the intracellular osmotic pressure at a relatively constant level which is higher than that of the environment. This theory maintains that excess water, diffusing through the outer membrane into the hypertonic cell, is extracted, isolated into the vacuole, and thus is eliminated when the vacuole is discharged (Kitching, 1938). However, Mast and Hopkins (1941) have shown that the marine *Amoeba mira* (*Flabellula mira* Schaeffer), which can withstand enormous changes in the

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concentrations of the salts of sea water, does not maintain a constant intracellular osmotic pressure. The intracellular pressure varies with that of the environment. They have shown that, if the amoeba is living in, and is adjusted to a medium, the intracellular pressure is always only slightly higher than that of the environment. Thus, a change in the osmotic pressure of the environment results in a corresponding change in the intracellular pressure.

In view of the fact that *Amoeba mira* is a marine organism and does not form contractile vacuoles even in dilute media, it was considered important to extend the investigations to a fresh water amoeba, which forms and eliminates contractile vacuoles, in order to ascertain whether these forms actually maintain a constant osmotic pressure by means of the action of the contractile vacuoles. The amoeba selected was a fresh water form collected from a swamp near Chicago. A single amoeba was isolated and allowed to multiply on an agar culture, using the methods of Rice (1935). All experiments were performed on the progeny of this amoeba.

METHODS AND RESULTS

Description of the amoeba

As in the case of most amoebae, it is difficult to decide upon its correct name. Figure 1 is a composite diagram illustrating its structure as observed in fresh water. Dujardin's *Amoeba lacerata* (1841) fits it fairly well. We shall then adopt the name of *Amoeba lacerata* Dujardin. Schaeffer (1926) has placed amoebae of this type in the genus *Mayorella* Schaeffer. Thus, using the system of Schaeffer, the name is *Mayorella lacerata* Dujardin.

Amoeba lacerata is a small amoeba, rounds up readily when disturbed, and in that condition has an average diameter of fifteen micra or a volume of about 1500 cubic micra. It varies from a limacine to a flattened fan shape and has a clear hyaline border (Fig. 1a) which is thick anteriorly and very thin posteriorly. A few, fine, pointed, indeterminate pseudopodia may project from the surface—anteriorly, superiorly, and posteriorly. Cytoplasmic granules are numerous—(1) an abundance of small, barely visible granules and (2) larger granules (or vacuoles) which are less numerous and more variable in number and size (Fig. 1b). The living nucleus (Fig. 1d) is spherical with a central endosome. Food is engulfed into the under surface as the amoeba moves over the food particles. Except in rare cases, food is engulfed with little or no water. Ingested food particles coalesce with clear vacuoles (Fig. 1c) of the cytoplasm, and the resulting small food vacuoles coalesce with each other as they do in *Amoeba mira* (Hopkins, 1938) to form large food vacuoles (Fig. 1f). These vacuoles are readily stained with Nile blue sulfate and neutral red. Egestion of food vacuoles (Fig. 1g) occurs at the posterior tip.

No direct connection or relationship between the food vacuoles and the contractile vacuoles has been observed except that occasionally a food vacuole may be ruptured into the contractile vacuole instead of to the outside. At egestion, the food vacuoles are rarely large and watery. Just before egestion they may be observed to decrease slowly in size until little more than food residues remain. This would indicate a protoplasmic absorption of water from the food vacuoles. It is also interesting to note that at the time when this apparent absorption takes place, the food vacuoles are located posteriorly and in close proximity to the region of

contractile vacuole formation which MacLennan (1941) and others have called the nephridioplasm. It is entirely possible that the water leaving the food vacuoles is absorbed into the contractile vacuoles.

The contractile vacuoles

In an amoeba moving forward rapidly, the contractile vacuoles form at the posterior end, as shown in Figure 1*c*. When the individuals are flattened out and feeding actively, it is difficult to distinguish the posterior end unless the location

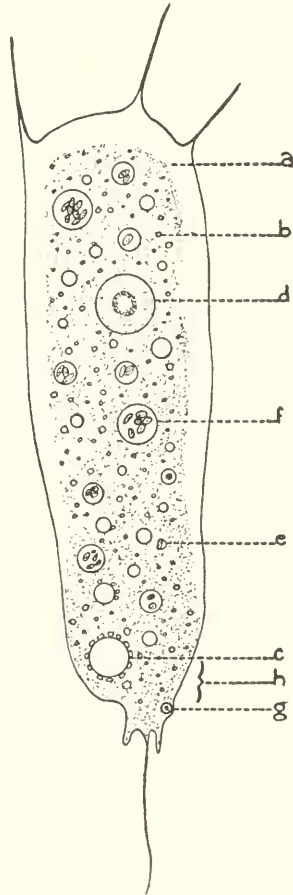


FIGURE 1. Diagram showing the characteristic structure of *Amoeba laccrata* Dujardin. *a*, hyaline border; *b*, large cytoplasmic granule (vacuole); *c*, contractile vacuole; *d*, nucleus; *e*, small clear vacuole adhering to a recently engulfed bacterium; *f*, food vacuole; *g*, food vacuole in position for egestion; *h*, nephridioplasm.

of the contractile vacuoles is accepted as a distinguishing feature of the posterior part of the cell. Observations on the origin of contractile vacuoles in active amoebae have revealed the following interesting facts:

(1) They develop in the posterior third of the cell, or, if they begin to develop in an apparently anterior part of the cell, that part will soon become the posterior part—as evidenced by change in the direction of protoplasmic streaming. If the posterior region of the amoeba becomes separated into two parts by a large food vacuole, contractile vacuoles will arise and be expelled independently on both sides. Pressure on the amoeba by a coverslip will cause contractile vacuoles to arise and be expelled independently in several parts of the cell. This phenomenon was also observed by Howland (1924b) in *Amoeba verrucosa*. A freely moving amoeba, as a rule, has only one formative region for contractile vacuoles.

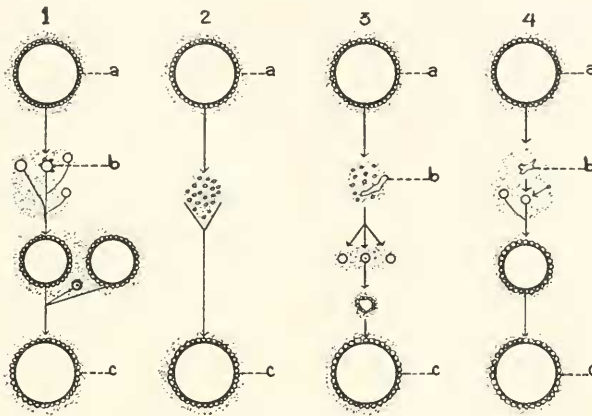


FIGURE 2. Diagrams showing contractile vacuole cycles, 1, 2, 3 and 4. Vacuoles *a*, are expelled to outside leaving residues, *b*, which by coalescence with other vacuoles, small and large, and swelling, form vacuoles, *c*, which are expelled.

(2) The nephridioplasm (Fig. 1h) is in the region in which plasmogel is rapidly becoming plasmosol. This region is often under considerable pressure (Mast, 1926). This is evidenced in *Amoeba lacerata* by the observations that the contractile vacuoles are often squeezed into oblong shapes, that food vacuoles and contractile vacuoles which normally never coalesce are sometimes squeezed so tightly together that the food vacuoles rupture violently into the contractile vacuole. The contraction of the contractile vacuole is, however, not always violent. Sometimes the contraction is very slow, while at other times, it may be only partial.

(3) While the contractile vacuoles increase in size by absorption, as will be shown later, the most conspicuous methods of growth are the coalescence of small droplets or vacuoles with one another, of large vacuoles with each other, and of small vacuoles with large vacuoles (Fig. 2). Small vacuoles less than 0.5 micron in diameter—can be observed coalescing with large vacuoles whose diameters are many times greater. Careful focusing on the outer boundary of a large contractile vacuole reveals this phenomenon. The coalescence of two contractile vacuoles is seldom violent. It occurs at about the same speed as the coalescence of two drops of olive oil.

(4) When a vacuole contracts, its contents are expelled to the outside. This occurs usually when the vacuole has a diameter of about 4 micra, but often smaller vacuoles contract, and larger ones may be formed before contraction occurs. Rup-

ture usually takes place through the postero-superior surface of the cell. Sometimes the contracting vacuole leaves no residue, but usually a small residue (Fig. 2*b*) remains, which coalesces with other small vacuoles forming new, large vacuoles. The residues are usually spherical, but often may assume oblong, stellate, or other shapes. Whether a residue remains or not, the region from which the previous vacuole was expelled contains small vacuoles which coalesce to form a large vacuole (Fig. 2*c*). These small droplets, or vacuoles, are continually appearing in the nephridioplasm. The region is generally devoid of food vacuoles.

The diagrams in Figure 2 illustrate four sequences which have been observed to occur in the nephridioplasm.

(5) All efforts to associate the contractile vacuoles with cytoplasmic granules of any sort have been futile.²

Tolerance of Amoeba lacerata for changes in the concentration of the medium

The swamp water from which the amoeba was collected was very dilute. The amoebae were first cultured in water from Lake Michigan to which grains of wheat were added. Attempts to culture it in various dilutions of sea water proved quite successful. It can be transferred, without deleterious effects, directly from lake water cultures to 5 per cent sea water to which grains of wheat are added. Excellent cultures of healthy amoebae develop within five days. Direct transference of the amoebae from 5 per cent sea water cultures to similar cultures made up in various dilutions of sea water results in excellent growth, even when the percentage is as high as 50 per cent. Direct transference to cultures having percentages higher than 50 per cent results for the most part in complete failure or very slow growth. If, however, the amoebae are first cultured in 50 per cent sea water and then transferred to 75 per cent or 100 per cent, survival and growth result. Slow growth may even be obtained in 125 per cent by adjusting the amoebae to 100 per cent and then transferring them to 125 per cent sea water.

The volume of the amoebae and the concentration of the culture medium

A study of the relation between the volume of the amoebae and the concentration of the culture medium in which they were grown has been made. The amoebae for this study were all from the same 5 per cent sea water culture and were inoculated into cultures of varying concentrations from one per cent to 75 per cent sea water. To each culture five sterile grains of wheat were added. Five cultures were set up at each concentration in exactly the same way and at the same time. When abundant growth and reproduction had occurred in all the cultures the amoebae were caused to round up by pipetting the culture fluid back and forth. Their diameters were then measured under the microscope with an eyepiece micrometer. At least 100 amoebae were measured for each concentration. The age of the cultures was the same at the time of measurement. A total of over 1,750 amoebae were measured. The results are presented in Figure 3, in which the average volume is plotted against the concentration of the culture. The increase in volume with an increase in concentration appears significant statistically. (The standard errors are graph-

² The very small vacuoles which form the larger contractile vacuoles undoubtedly have been mistaken for granules by various authors.

ically indicated in the curve by the length of the vertical lines drawn through the points.) The total range of variation in average volume is, however, only a little over $750 \mu^3$. This increase in volume with concentration, if significant, is probably due to a lower rate of fission in respect to growth in higher concentrations than in lower concentrations. It should be mentioned here, however, that much greater

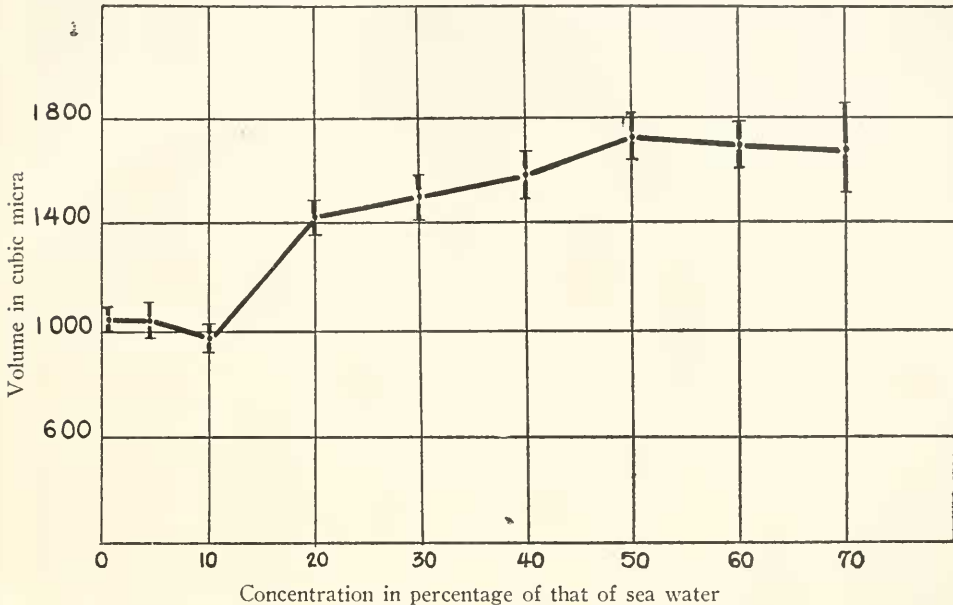


FIGURE 3. Graph showing relation between volume of amoebae and the concentration in which they are grown.

variations occur in average volume of amoebae from cultures when the nature of food is allowed to vary more than was the case in this series of cultures. (See average volumes of cultures of Figure 4.)

Relation between the protoplasmic osmotic pressure and osmotic pressure of the external medium

In amoebae which have become adjusted to 50 per cent sea water, is the cytoplasmic concentration the same, higher, or lower than that of amoebae adjusted to 5 per cent or 20 per cent sea water? What relation does the cytoplasmic concentration bear to the concentration of the culture medium?

Since these amoebae round up into spheres when agitated, the osmotic concentration of the protoplasm can be readily approximated. When the amoebae are placed in sea water of a given strength with a resulting decrease in volume, we can safely conclude that the osmotic pressure is higher externally than internally. If, on the other hand, the volume of the amoebae increases, the osmotic pressure of the protoplasm is greater than that of the medium. If, however, the plasmalemma is permeable to the solutes of the medium the permanence of the change will depend upon the degree and speed of solute penetration.

The results of experiments designed to answer the above questions were as follows:

Amoebae raised in 5 per cent sea water, and consequently adjusted to it, are placed in one per cent sea water. They immediately swell to a maximum volume and then gradually decrease in volume until approximately their original volume is regained. The smaller the change in concentration, the more rapid is the return to original volume. The same is true when amoebae cultured in any strength in which they will live are placed in higher and lower concentrations.

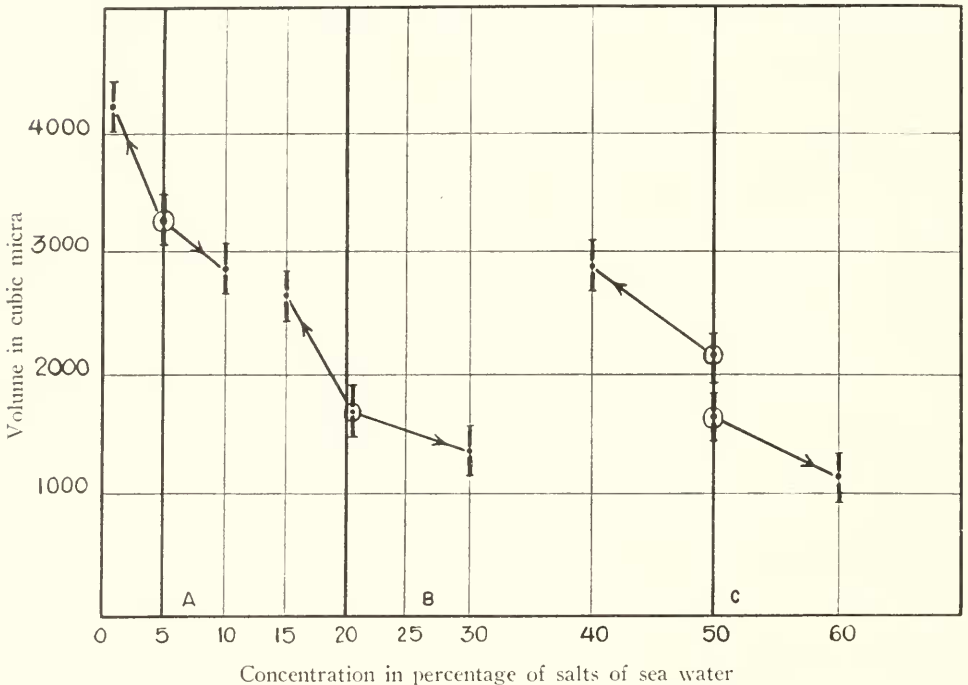


FIGURE 4. Graph showing volume change occurring immediately when amoebae grown in various percentages of sea water are transferred to lower and higher percentages respectively. *A*, amoebae grown in 5 per cent transferred to one per cent and 10 per cent; *B*, grown in 20 per cent and transferred to 15 per cent and 30 per cent; *C*, grown in 50 per cent and transferred to 40 per cent and 60 per cent.

The results of a statistical study of the initial changes in volume when the concentration of the medium is increased or decreased from that of the culture medium are given in Figure 4. In this experiment the amoebae were cultured in 5 per cent, 20 per cent, and 50 per cent sea water (Fig. 4*A*, *B*, and *C* respectively). The amoebae were agitated in the culture; the diameters of from fifty to one hundred individuals measured; the volumes calculated and averaged. The concentration was increased on some and decreased on others; and then the volumes of fifty amoebae whose medium was concentrated, and fifty amoebae whose medium was diluted were measured and averaged. Measurements of volume were all made between 5 and 30 minutes after the concentration was changed. In the figure, the

circles give the average volume of the amoebae in culture medium. The other points represent the average volume of amoebae which have been transferred to the concentration indicated. Volume is indicated on the ordinates. The concentration of the culture is indicated on the abscissas. The length of the vertical lines through the points represents the extent of the standard error.

These results indicate very definitely that the osmotic pressure of the protoplasm of *Amoeba lacerata* varies in the same direction as that of the external medium as was found to be the case in *Amoeba mira* (Mast and Hopkins, 1941). The osmotic pressure of protoplasm of amoebae cultured in 5 per cent is not over that of 10 per cent, nor under that of one per cent sea water. The osmotic pressure of those cultured in 20 per cent sea water is not over that of 30 per cent nor under that of 15 per cent. Finally the osmotic pressure of those cultured in 50 per cent sea water is not over that of 60 per cent nor under that of 40 per cent. Due to the rapid return of the amoebae to their original volume when small concentration changes were made, we were unable to make more accurate measurements of protoplasmic concentration. Therefore, we are unable to say definitely whether the adjusted amoebae were isotonic, slightly hypotonic, or hypertonic to the culture medium. Since the amoeba can live as well in 50 per cent sea water as it can in 5 per cent, and since the protoplasm of amoebae living in 50 per cent assumes a concentration greater than that of 40 per cent sea water, a wide range of protoplasmic concentrations appears to be compatible with the metabolic processes.

Factors affecting the activity of the contractile vacuole system

Contractile vacuoles form and are expelled in all concentrations of sea water in which the amoebae will live and reproduce. The rate of formation and expulsion is, of course, slower in concentrated than in dilute culture media. On encystment, growth and expulsion of contractile vacuoles cease and are resumed when excystment occurs. Kitching (1938) has shown that the oxygen tension of the medium is an important factor in the activities of the vacuoles of some protozoa, including various Peritrichs, *Amoeba proteus*, and *Amoeba mira*. During the present experiments, it has been observed that if the amoebae are sealed under a coverslip and left for some time, the rate of vacuolar expulsion is invariably retarded. On the other hand, if fresh aerated culture fluid is continuously circulated under the coverslip, the vacuoles remain active. This would suggest that oxygen is important in the activity of the contractile vacuoles. More precise experiments on the effect of oxygen are necessary.

A study of the effect of the hydrogen ion concentration of the medium was made. Sudden increases and decreases cause an increase in the rate of vacuolar activity. On the other hand, if amoebae are adjusted to and living at a given pH, there is no consistent relation between the pH of the medium and the rate of fluid elimination.

The effect of the concentration of the culture medium on the activity of the contractile vacuoles

An extensive study was made of the relation between the concentration of the culture medium and the rate of fluid elimination by means of the contractile vacuoles. In view of the fact that uncontrollable factors act to affect the activity of the

vacuoles, the study had to be statistical. For these experiments amoebae were inoculated into cultures made by adding six grains of wheat to various percentages of sea water in Petri dishes. The rate of elimination was measured only after adjustment evidenced by growth and reproduction was complete. Measurements were made of the rate in amoebae living in 5, 10, 15, 20, 25, and 50 per cent sea water. As we have seen, this amoeba flourishes equally well in all of these concentrations.

The rate of fluid elimination was measured as follows: A coverslip was cleansed and dropped onto the bottom of a culture dish; the culture fluid was agitated with a pipette and the amoebae were allowed to settle and attach themselves onto the coverslip. When attachment had occurred, the coverslip was then inverted and placed on a slide between two parallel streaks of vaseline. The streaks of vaseline served to stabilize the coverslip, prevent the amoebae from being crushed between slide and cover, and allow free circulation of water underneath. During an experiment, fresh aerated sea water of the same strength as the culture medium of the particular amoebae being observed was continuously added at one end of the cover and drawn out at the other by means of a strip of filter paper. Observations were made under an oil immersion lens. The diameters of vacuoles were measured with a micrometer eyepiece and the volumes calculated. The vacuole was always measured just before expulsion. The fluid elimination of an amoeba for a ten minute interval was obtained by adding the volumes of the vacuoles formed during that time. The amoeba was then mechanically agitated, causing it to round up, and its volume obtained by measuring its diameter while thus rounded. The rate was calculated in terms of the cubic micra eliminated per minute by 100 cubic micra of protoplasm.

The results of this study of the rate of elimination of amoebae living and reproducing in various concentrations of sea water are presented in Figure 5. The rates of over one hundred amoebae were measured to obtain the curves in this figure. The rate of each amoeba measured is represented by a point. The average rate of all the amoebae measured in each percentage of sea water is represented by a square, while the highest rate obtained for each concentration of medium is represented by a circle. The average rates have been connected by a curve, as have the maximum rates. A third curve has been drawn whose shape is determined by the formula: $RC = K$, where R is the rate of elimination, C is the concentration of the medium to which amoebae are adjusted, and K is a constant.

If we take into consideration cysts whose rates are zero and which are not included in the figure, we find that the rates of amoebae grown in any concentration may vary from zero to a maximum. The maximum rates vary inversely with the concentration. Neglecting those amoebae, or cysts, whose rates are zero, the average rates also vary inversely with the concentration. Neither the curve for the maximum rates, nor that for the average is exactly parallel with the curve expressed by the equation $RC = K$, but the parallelism in both cases is sufficient to justify the conclusion that under ideal conditions in which concentration is the only variable, the equation would express accurately the relationship existing between the concentration of the medium and the rate of elimination.

Kitching (1938) has pointed out that if a high unalterable protoplasmic concentration was maintained by the action of the contractile vacuoles, the rate of

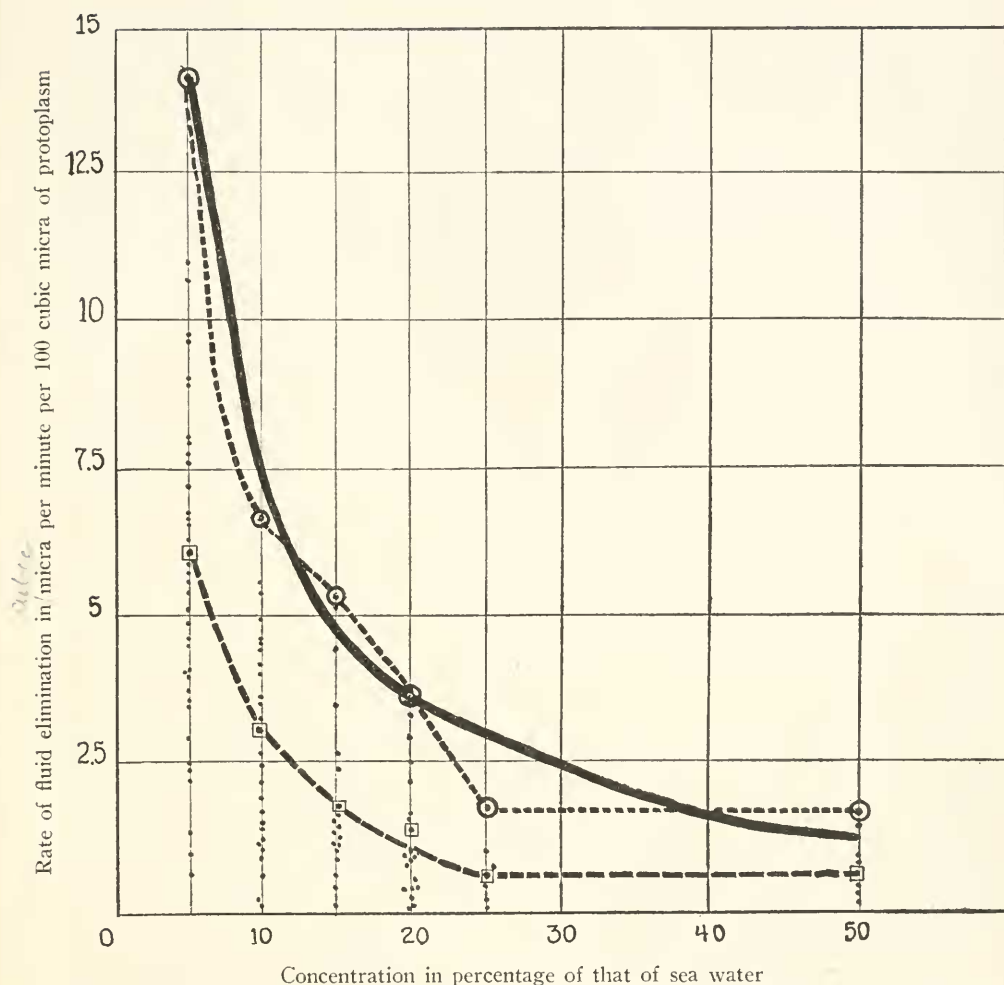


FIGURE 5. Graph showing the relation of the rate of fluid elimination to the concentration of salts in sea water. The amoebae were tested in the concentration in which they were grown. Squares, average rates; circles, maximum rates; dots, individual rates. Solid line is curve for equation; R (rate) $\times$ C (concentration) = K (constant).

elimination should be inversely proportional to the external concentration, and that the curve should be a straight line whose slope is constant instead of a curve whose slope increases as the external concentration decreases. The curves obtained by Kitching (1938) for *Peritrichs*, those obtained by Mast and Hopkins (1941) for *Amoeba mira*, and those obtained here, never approach a straight line. The actual rates are either always too low in the intermediate concentrations or too high in dilute and concentrated solutions, depending of course upon the rate which is assumed to be correct. Therefore, in view of these facts, it is not possible that the correct curve is a straight line.

The effect of changing the external concentration on the volume of individual vacuoles

(A) Vacuoles remaining in the amoeba: When an amoeba with a large contractile vacuole is adjusted to 5 per cent sea water and is placed suddenly in 100 per cent sea water, the amoeba shrinks. By careful observation it can also be observed that the contractile vacuole shrinks. The membrane of a vacuole for a while shows sufficient elasticity to maintain a spherical shape, but the shape is not maintained. As more and more water is drawn out of the vacuole, the membrane collapses (see Fig. 6).

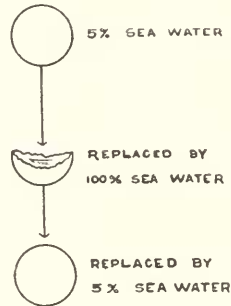


FIGURE 6. Diagram illustrating the collapse of a contractile vacuole when subjected to hypertonic sea water, and its recovery when placed in sea water to which it originally was isotonic.

If the amoeba, now in 100 per cent sea water with the vacuole in the condition shown in Figure 6, is returned to 5 per cent sea water, the amoeba and the collapsed vacuole both absorb water and swell. The vacuole soon regains its original volume and may even become larger. It would appear from this experiment that the vacuole is acting as a simple osmometer. When the hypertonic sea water was added, the water was extracted from the vacuole but not the dissolved substances. Consequently, when dilute sea water was again added this substance acting osmotically drew water back into the vacuole.

(B) Vacuoles removed from the amoeba: In the preceding experiment, it could still be argued that the swelling of the vacuole was due to a secretory process requiring oxygen (Kitching, 1938), since the swelling of the vacuoles took place in the amoeba and in the presence of the cellular activities. If, however, the vacuole was removed from the cell and suspended in the medium free of all contact with protoplasm the influence of other cell parts would be eliminated.

By crushing amoebae under a coverslip while watching the vacuoles closely it was observed that the vacuoles do not always disintegrate; some of them remain intact after they are liberated, free of protoplasm, into the medium. When the vacuoles are thus exposed to the outside medium they swell noticeably, indicating that the elastic forces of the cell were inhibiting swelling to some extent. When such isolated contractile vacuoles from amoebae adjusted to 5 per cent sea water are changed to 100 per cent, they shrink. Then, on being returned to 5 per cent sea water, they swell just as they do while still in the amoebae. *It is obvious then that they act as simple osmometers; that their contents contain osmotically active sub-*

stances in solution, and that the swelling of the vacuoles does not depend upon other protoplasmic processes.

The adjustment of Amoeba lacerata to changing concentration

We have described the immediate effects upon the amoeba and contractile vacuole of changing the concentration of the medium. The story is not completed, however, until we have described the complete series of changes in volume and in the rate of elimination which occur during the adjustment process. Observations have been made upon the volume and vacuolar activities during the adjustment process, both when amoebae cultured in 5 per cent sea water were changed to 50 per cent and when amoebae cultured in 50 per cent sea water were changed to 5 per cent.

(I) Adjustment of amoebae cultured in 5 per cent sea water to 50 per cent sea water: When an actively moving amoeba, raised in 5 per cent sea water, is changed to 50 per cent sea water and allowed to remain, it rounds up and its contractile vacuole shrinks greatly. The amoeba remains for some time in the inactive spherical condition. The contractile vacuole increases in volume very slowly. Repeated measurements of the diameter of the amoeba itself reveals the fact that it increases slowly in volume. This increase continues until it has regained approximately the value it had in 5 per cent sea water. As soon or soon after it has regained this original volume, protoplasmic streaming becomes more definite, attachment to the substratum is made, and normal locomotion begins. The rate of elimination of fluid by the vacuole remains at a low level. In Figure 7*A* and *B* are plotted curves showing the observed changes in protoplasmic volume and the rate of elimination of two amoebae when they were suddenly changed from 5 per cent sea water in which they had been cultured to 50 per cent sea water. In these experiments the rate of fluid elimination by the contractile vacuoles was measured, 50 per cent sea water added, and the volume and rate measured again. Since the rate of elimination was so extremely slow it was possible to make only one measurement before adjustment was completed, but the diameter of the amoeba was measured at frequent intervals until adjustment was complete and locomotion resumed.

(II) Adjustment of amoebae cultured in 50 per cent sea water to 5 per cent sea water: In Figure 7*C* and *D*, we have plotted curves showing observed changes in volume and the rate of elimination when amoebae were suddenly changed from their 50 per cent sea water culture to 5 per cent sea water. In these curves it will be observed that (1) the amoebae swell and then shrink until their original cultural volume is regained, (2) the rate of elimination increases rapidly to a high value which may either remain high, or after an initial increase may fluctuate, and (3) that the time required for the amoebae to regain their original volume may be long or short.

In this experiment it appears quite possible that the activity of the contractile vacuole may have been helpful in bringing the volume of the amoeba back to normal. But we already know from experiments that when adjustment is complete the amoebae will now shrink if placed in 10 per cent sea water. Therefore, while the activity of the contractile vacuole may have helped the amoebae to regain their

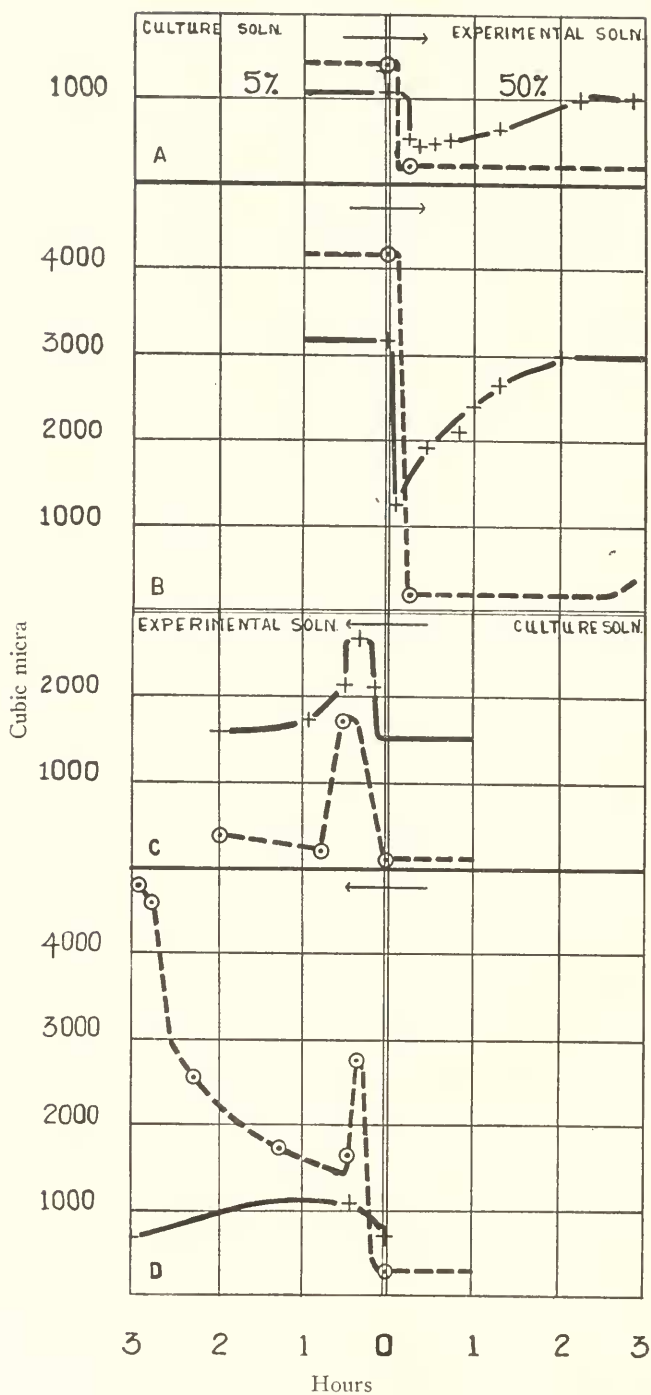


FIGURE 7.

normal volume, the protoplasmic concentration at the same time has dropped from a value approximating that of 50 per cent sea water to a value approximating that of 5 per cent sea water.

DISCUSSION

Krogh (1939) after reviewing the works of various authors on the functions of the contractile vacuoles of the Protozoa, concluded that the evidence was predominantly in favor of the "osmoregulatory theory," which holds that these vacuoles serve to maintain the internal protoplasmic concentration at a constant level which is higher than that of the outside medium. In view, however, of the work of Buchthal and Peterfi (1937) who found only slight potential differences existing between the protoplasm of *Amoeba sphaeronucleus* and the outer medium, Krogh concludes his discussion with a "warning against too schematic conceptions." He says further, "It seems possible that the contractile vacuole may come much nearer in its function to the kidney of higher animals than is indicated by direct studies of osmotic regulation."

The contents of the contractile vacuoles

Weatherby (1927) was unable by means of very delicate tests to show the presence of urea or ammonia in the contractile vacuoles of paramecia. Kitching (1938) points out that the maintenance of a constant concentration higher than the outer medium would require the elimination of fluid more dilute than the protoplasm. He believes from his experience that it is entirely possible that these vacuoles contain only distilled water. Ludwig (1928) argues that carbon dioxide is eliminated by way of the contractile vacuoles, but without evidence. The possibility of the elimination of carbon dioxide, carbonic acid, and carbonates by the contractile vacuoles, however, has not been disproven. Various authors have maintained that granular anlagen give rise to contractile vacuoles (Lloyd and Scarth, 1926; Taylor, 1923; Howland 1924a; MacLennan, 1933). Hopkins (1938) and Mast and Hopkins (1941) maintain that the food vacuoles of the marine amoeba, *Amoeba mira*, in addition to being the seat of digestion, act as do contractile vacuoles and that the anlagen of these vacuoles may contain granules. They also show that the substances that make up these granules may never aggregate in the form of granules but remain in solution in the vacuoles or their anlagen. The present observations on *Amoeba laccrata* do not show definite connections between granules of the cytoplasm and the contractile vacuoles or that their anlagen contain granules, but they do show that they contain osmotically active substances.

The concentration of the protoplasm

The actual concentration of the protoplasm has never been ascertained. When we speak of the osmotic strength or activity of a cell we can of necessity refer only to the attraction of the cell for the solvent and compare the strength of this attrac-

FIGURE 7. Graphs showing changes in volume and rate of fluid elimination of individual amoebae when they were transferred from the percentage of sea water in which they were grown to lower or higher percentages. *Solid curves*, volume changes; *broken curves*, changes in rate of fluid elimination. Arrows show the direction of the concentration changes. *A* and *B* from 5 per cent to 50 per cent sea water; *C* and *D* from 50 per cent to 5 per cent sea water.

tion to the attraction exerted by known solutions. A cell is said to be isotonic to a solution of known strength when the affinity of the cell and the solution for solvent are equal; i. e., a further increase in the strength of the solution would shrink the cell. No deductions can be made as to the total molecular concentration of the cell components. The osmotic concentration, the intermolecular attractions and repulsions of the protoplasmic molecules and tendencies of the protoplasm to become hydrated are all involved. The tensile or elastic strength of the protoplasm, membranes, and cell wall all work in opposition to the osmotic concentration and hydration. The shrinkage or swelling of a cell depends upon a summation of these inside forces. Most cells are in equilibrium with their environment; i. e., the summation of forces inside equals those outside. The cells forming contractile vacuoles are said to be exceptions according to the osmosis-secretion theory of Kitching (1938). Cells forming contractile vacuoles live in media much more dilute than solutions necessary to plasmolyze them. Such cells must be absorbing water continuously from their more dilute outer medium and continuously bailing out fluid, more dilute than the protoplasm, by the action of the contractile vacuole. Even though the concentration of the protoplasm is higher than the outside medium, this does not follow. The elastic or tensile strength of the membrane and ectoplasm or plasmagel, and for that matter intermolecular attractions of the protoplasmic molecules must exert considerable opposition to the entrance of water. The results here presented and those of Mast and Hopkins (1941) show that *the osmotic activity involved in collecting water eliminated by the contractile and food vacuoles is resident in the vacuoles themselves not in the surrounding cytoplasm*. The surrounding cytoplasm serves merely as a membrane through which the water is drawn. It is also clearly seen from these results that we cannot speak accurately of the osmotic activity of the protoplasm in general. Only of specified parts which have no internal differentiation can we hope to accurately be specific as to osmotic activity.

The concentration of the protoplasm of *Amoeba lacerata* is only slightly different from the surrounding medium. The present experiments demonstrate this fact. (see Figures 4 and 7). The results presented in Figure 5 are consistent only if we assume that the concentration of the protoplasm outside of the vacuoles varies in the same direction as the medium. If we also assume that the protoplasm by oxidative or other metabolic processes gives rise to a constant supply of metabolic by-products for isolation into the vacuoles which swell and coalesce in a constant manner (which is true in the average amoeba), then the curve of Figure 5 is explained perfectly. It is unfortunate that the experiments on the rate of elimination could not be continued below 5 per cent sea water. The reason for this inability was the extreme variability of rates obtained. In other words, in extremely dilute media, the rate of elimination showed little or no dependence upon the osmotic pressure of the external medium. This leads to the conclusion that in extremely dilute media, the osmotic pressure of the external medium is of little consequence in determining the rate of fluid elimination.

The function of the contractile vacuoles

If water is absorbed mainly by the cell due to the osmotic activity of the contents of the contractile vacuoles, the primary function of the vacuoles is not the elimination of water. *The water eliminated by the vacuoles is only water absorbed as a*

consequence of their own contents. The osmotically active contents of the vacuoles are discharged to the outside and are wasted. These wastes are in all probability metabolic by-products. They could be incompletely oxidized food, carbonic acid, carbonates, ammonia, urea, uric acid, or other waste products.

Kitching (1938) has shown that activities of contractile vacuoles and volumes of various protozoa are controlled by the oxygen tension. He takes this fact to mean that oxidative energy is utilized in extracting water from the protoplasm and the secretion of this water into the vacuoles. He argues that energy is needed since this is accomplished against an osmotic gradient. He also finds that cyanide inhibits the action of the contractile vacuoles in these forms and that coincident with cyanide inhibition, the body volume increases. When cyanide is removed, the contractile vacuoles resume their activity and the cell volume returns to normal. His interpretation of these facts is that the removal of oxygen or the addition of cyanide inhibits oxidation which in turn deprives the vacuoles of energy and they cease extracting water from the protoplasm; but meanwhile the cytoplasm continues to absorb water from the medium thus swelling the cell. He omits any consideration of the effect of cyanide on protoplasm except the inhibition of oxidation. His interpretation does not take into consideration the following possibilities: (1) While the relation of oxygen tension to vacuolar activity is demonstrated, this relation could be as well explained by the formation of osmotically active substances in vacuoles as a result of oxidation. (2) Cessation of the oxidative process may initiate many changes in the protoplasm, for example, its degenerative breakdown which would cause swelling. Kitching's own results, the results of others, and the present results are more consistent with the conclusion that the contractile vacuoles function to eliminate metabolic wastes, and that the elimination of water is merely a consequence of the osmotic activity of these wastes.

When one stops to consider the possibility of the formation of vacuoles more dilute than the protoplasm, it is seen to be an impossibility and contrary to known physico-chemical laws for the following reasons. For it to be possible, water would have to be isolated and separated against solution and chemical forces. This water would have to be isolated into regions of equal hydrostatic pressure by some force which cannot exist since water has been attracted into the protoplasm by exactly opposite forces. There are two ways by which water can be separated from protoplasm, i.e., setting up forces which attract or repulse water more strongly than protoplasm. (1) By chemical changes occurring in regions of the protoplasm, for instance, oxidation which results in localized increases in chemical and osmotic forces (vacuoles). (2) By changes in protoplasm in general. Energy resulting from the oxidation of food can be used in polymerization whereby the forces leading to hydration are lost. This releases water from combination with the protoplasm. The water would tend to collect in localized regions, but during this collection, waste products become dissolved and consequently, the vacuoles so formed would contain waste and salts, not distilled water. Oxidation in the protoplasm enhances the water-combining powers of some substances, but not all.

If the vacuoles contain a solution whose osmotic pressure is less than that of the surrounding cytoplasm, then water will be drawn back into the cytoplasm and not expelled to the outside. This statement, however, would not be true if the vacuole membrane were impermeable to water.

In the experiments concerned with isolated vacuoles, it has been clearly shown that the internal contents of the vacuoles do have an osmotic pressure and that the vacuole membrane is permeable to water. *The osmotic pressure of the vacuoles is either equal to or greater than that of the cytoplasm.*

The relation between the osmotic activity of the protoplasm as a whole and that of the external medium in which the amoebae live

We have demonstrated that the osmotic activity of *Amoeba lacerata* varies directly with that of the environment. This also is true of *Amoeba mira* (Mast and Hopkins, 1941) and of the peritrichs investigated by Mast and Bowen (1945). In addition, it appears that *Amoeba proteus* (Mast and Fowler, 1935), peritrichs (Mast and Bowen, 1945), plant cells (many authors) and many other cells maintain a higher osmotic pressure than their natural medium. This is the cause of turgidity. In the protozoa with contractile vacuoles as well as other animal and plant cells, the extent of this hypertonicity of a cell over its environment must depend upon the strength of the membranes, and other ectoplasmic structures. The external medium remaining constant, some of the factors that would cause swelling of cells are: (1) weakening of cell boundaries, (2) peptization of protoplasmic proteins, (3) increase in cellular anabolism without increase in cellular excretion, or (4) merely failure of the excretory processes.

The extent of the difference in osmotic pressure existing between the cell contents and the outside medium has not been demonstrated either for *Amoeba mira* or *Amoeba lacerata*, but if we include consideration of the osmotic pressure of the vacuoles, then the difference is appreciable but variable, being higher inside than outside.

Factors determining the rate of vacuolar output

In a given protozoan, the rate of fluid output depends on a number of factors. These factors and how they operate are not clearly defined but the following undoubtedly are involved:

(1) Respiration. The work of Kitching has demonstrated this. If the basic osmotic pressure of a cell is in equilibrium with that of the environment, the oxidative (aerobic or anaerobic) breakdown of foodstuffs will result in an increase in the osmotic activity of the cell as a whole even though these by-products are probably isolated into the vacuoles immediately by the formation of a precipitation membrane. It is readily seen from the facts presented here and by Mast and Hopkins (1941) that the rate of fluid elimination would depend upon the respiratory rate. Condensation of the by-products in the form of condensation granules would decrease their osmotic activity. While the rate of oxidation does have this controlling influence upon the rate of fluid elimination, the shape of the curve of Figure 5 is not determined by variations in oxidative rate. The oxygen supply, of course, was not absolutely constant but reasonably so. Since the curve represents an average, the variations are accounted for statistically.

(2) Food. In protozoa, food taken into food vacuoles and digested there adds to the over all osmotic activity of the cell, but if digested food is rendered insoluble by condensation into crystals or other food reserve bodies, this osmotic increase is nullified. This storage may not take place before appreciable increase in intake of

water has been affected. Also, the water engulfed with food would naturally have an influence on the rate of fluid uptake, but not upon the output as suggested by Kitching (1939).

(3) Agents causing polymerization or breakdown of substances in the protoplasm.

(4) Changes in osmotic activity of outer medium will cause increases or decreases in water intake and consequently the output. The permanence of these effects will depend upon the permeability of the membrane to the substances in the external medium.

Two vacuole systems as opposed to one

Amoeba mira (Hopkins, 1938) forms only one system of vacuoles. By this statement it is meant that all vacuoles which arise in this amoeba coalesce to form large cloacal vacuoles, which are eliminated in order of formation. The functions of excretion and digestion are accomplished by this system. On the other hand, *Amoeba lacerata* forms two entirely different sets of vacuoles: one which goes to make up the contractile vacuoles, and is excretory in function while the other system goes to combine with the engulfed food and is digestive in function. All protozoa probably can be separated into two groups: one primitive group, having only one vacuolar system; and a specialized group, having two systems.

SUMMARY

1. *Amoeba lacerata*, a fresh water amoeba, is able to adjust and live in any concentration of the salts of sea water up to 125 per cent.

2. Contractile vacuoles are formed in all concentrations and are separate from food vacuoles.

3. Food is engulfed with little or no water and small protoplasmic vacuoles coalesce with it. When digestion is completed, the food residues are eliminated with little or no fluid.

4. The major part of fluid elimination occurs by way of the contractile vacuoles.

5. Contractile vacuoles grow in size by coalescence and osmotic swelling.

6. The rate of fluid elimination is affected by a number of factors, but under constant optimal conditions it varies inversely with the concentration of the medium. It is suggested that the rate is proportional to the rate of catabolism.

7. The osmotic pressure of the protoplasm varies in the same direction as that of the outside medium. The protoplasm of completely adjusted amoebae is very nearly equal to that of the medium, the osmotic difference being less than that of 5 per cent sea water (0.13 atmosphere).

8. Contractile vacuoles have been shown to contain osmotically active substances; they shrink in hypertonic and swell in hypotonic solutions regardless of whether they remain in the cell or have been removed from the cell.

9. The volume of the amoeba is only temporarily dependent on the concentration of the medium. After adjustment, the volume shows no dependence upon concentration of the medium. If the amoeba is placed in a hypertonic solution the cell shrinks and then swells to its original volume within three hours time. Conversely, if placed in a hypotonic solution, it swells and then shrinks to its original volume.

10. The cell membrane is either permeable to substances in the medium especially when adjusting to a change in the concentration of the medium, or is capable of modifying its internal osmotic activity in some other way.

11. The contractile vacuoles are obviously excretory and also osmoregulatory organelles since they remove waste substances which would otherwise cause increases in the osmotic pressure in the cell.

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